NLRP3 Inflammasome Is a Target for Development of Broad-Spectrum Anti-Infective Drugs

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We describe the molecular mode of action and pharmacodynamics of a new molecular entity (NME) that induces the NLRP3 inflammasome-mediated innate immune response. This innate response reduces the pathogen load in an experimentally induced methicillin-resistant Staphylococcus aureus infection, enhances survival in an experimentally induced Gram-negative bacteremia, and overrides the escape mechanism of an obligate intracellular pathogen, viz. Chlamydia pneumoniae. Furthermore, the NME is more effective than standard-of-care antibiotic therapy in a clinically established multifactorial bacterial infection. Analysis of transcriptional regulation of inflammasome signaling genes and innate/adaptive immune genes revealed consistent and significant host changes responsible for the improved outcomes in these infections. These studies pave the way for the development of first-in-class drugs that enhance inflammasome-mediated pathogen clearance and identify the NLRP3 inflammasome as a drug target to address the global problem of emerging new infectious diseases and the reemergence of old diseases in an antibiotic-resistant form.

Despite the benefit of antibiotics, it now appears that antibiotics have selected for bacterial resistance factors and have given rise to the so-called “superbugs.” The CDC has reported that more people now die from hospital-acquired infections than HIV (23), and methicillin-resistant Staphylococcus aureus (MRSA) is among the leading causative pathogens. In the past, MRSA was a problem that was largely confined to hospitals and extended care facilities; however, emergence and spread of community-acquired MRSA (CA-MRSA) is reaching epidemic proportions (10, 14).

The development of new antibiotics has recently slowed considerably, in large part due to the long lead time from discovery to market for new molecular entities (NMEs), the high failure rates of new drug candidates, the short treatment period, and the low reimbursement rates for antibiotics. Thus, medical practitioners are left in an ever-widening chasm between emerging new antibiotic-resistant pathogens and a dearth of therapeutic alternatives. Given the recent evidence that bacterial pathogens harbor the resistance genes to all known classes of antibiotics (11), continued efforts to discover and develop new antibiotics are simply repeating the same experiment with the expectation of a different result.

Mammals have evolved tolerance mechanisms to accommodate beneficial microorganisms as well as potential pathogens and have evolved resistance mechanisms to reduce the pathogen burden during infection (reviewed in reference 33). Therefore, we have been exploring a strategy of modulating the host tolerance and resistance mechanisms as a means to ablate infection from progressing to disease.

The inflammasomes are the major regulator of resistance and tolerance in mammalian cells and are comprised of a family of cytosolic receptors, called NOD-like receptors (NLR), that are involved in innate immune recognition of pathogen-associated molecular patterns as well as intracellular and extracellular damage-associated molecular patterns (reviewed in reference 24). Thus far, more than 20 inflammasomes have been identified, and many are present in nearly every cell type. NLR protein 3 (NLRP3; also known as NALP3) is the most extensively studied inflammasome and has been found to be activated by a diverse range of stimuli, including microbe-derived products (8, 12, 18, 28, 35), environmental factors (6, 13, 22), and endogenous molecules (16, 25, 31, 39).

Herein, we show that regulation of the NLRP3 inflammasome is a safe and effective means to prevent Gram-positive and Gram-negative bacterial infections from progressing to disease (enhanced tolerance) and abrogate disease progression in established bacterial infection (enhanced resistance). The obvious advantage of this therapeutic strategy is that, by targeting the host tolerance and resistance mechanisms, selective pressure for the expression of pathogen resistance is greatly reduced or altogether absent.

Thacker and colleagues (34) reported the isolation, structure elucidation, and synthesis of an NME, 1-peptidyl-2-arachidonoyl-3-stearoyl-sr-glyceride, derived from a caprine serum fraction similar to that which had been previously reported (2, 20, 29, 30). The chemical structure is shown in Fig. 1 and here is referred to with the acronym pDAG. In the previous report it was also shown that the biological activity of pDAG was attributable to the 18-amino-acid peptide moiety that is here referred to as acALY18. In this report we describe the molecular mechanism of action (MMOA) of acALY18 as a first-in-class, broad-spectrum anti-infective agent targeting the NLRP3 inflammasome.

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MATERIALS AND METHODS

Details of the methods used are available in the supplemental material and are summarized here.

Animal care and use. Animals were handled in accordance with NIH policies for humane care and use of laboratory animals, and studies were conducted under specific protocols approved by the Institutional Animal Care and Use Committee of Lampire Biological Laboratories, Inc. (for antibody production and adjuvant experiments in rabbits), Covance, Inc. (for adjuvant experiment in rabbits and Salmonella bacteraemia experiments in mice), and Drexel University College of Medicine (for the MRSA study of mice).

Test article. pDAG was isolated and synthesized as previously described, and acALY18 was synthesized by solid-phase methods as previously described (34). FNN-21 was synthesized by solid-phase methods as previously described (37).

Cell culture. Normal primary human fibroblasts (GM5659) obtained from the Coriell Institute (Camden, NJ) were cultured in 70-mm dishes as previously described (5). Mouse fibroblasts were established from C57BL/6 (Jackson Laboratories) and NLRP3−/− and ASC−/− mice (gift from Genentech, South San Francisco, CA). THP-1 human monocytes from the American Type Culture Collection (ATCC TIB202; Manassas, VA) were propagated in accordance with the ATCC established protocol.

Gene expression. Fibroblasts and THP-1 monocytes were harvested, and RNA was purified using the Qiagen RNA isolation kit. Five micrograms of RNA was converted to cDNA and applied to the human innate and adaptive immune response RT2 Profiler PCR array and to the inflammatory array (SABiosciences). Gene expression was assayed using SYBR green as a reporter. The cycle threshold ($C_t$) of the genes was normalized to that of β-actin.

Cytokine and chemokine protein assay. Cytokines and chemokines in fibroblast supernatants were measured by enzyme-linked immunosorbent assay according to the manufacturer’s specifications (eBioscience). Cell lysates were assayed using the caspase-1 colorimetric assay kit (Abcam) according to the manufacturer’s protocol.

Tissue histology and bacteriology. The lesion tissue was collected using an 8-mm punch biopsy tool and weighed. Samples of the excised tissue for determining pathogen load were homogenized in 4 ml of sterile saline using an 8-mm punch biopsy tool and weighed. Samples of the excised tissue, and inflammatory infiltrate. Separate sections were deparaffinized with xylene, rehydrated, and stained with Masson’s trichrome. Immunostaining was performed using antibodies to α-smooth muscle actin (1:100, Abcam, Cambridge, MA), washed, then incubated with donkey anti-rabbit–Cy3 (1:1,000, Jackson Immunochemicals, West Grove, PA), counterstained with 4′,6-diamidino-2-phenylindole (DAPI), and viewed with an epifluorescence microscope. Images were taken at 100× and 400× magnification.

Monocyte activation. Primary normal human fibroblasts (GM5659; Coriell Institute) were transfected with or without 3 ng/ml acALY18 and cultured as previously described. Cells not receiving acALY18 were treated with Lipofectamine alone as a control. Human THP-1 monocytes were cultured in T75 flasks in RPMI plus 10% fetal bovine serum and 1% penicillin-streptomycin for 48 h and harvested after centrifugation (2,000 rpm for 5 min at 4°C). THP-1 monocytes were resuspended in the medium collected from the acALY18-treated or control-treated fibroblasts and incubated for an additional 72 h. In a separate experiment the harvested THP-1 monocytes were resuspended in the medium collected from the acALY18-treated or control-treated fibroblasts, added back to the respective fibroblast culture, and allowed to incubate for an additional 72 h. Monocytes were recovered from the respective cultures and centrifuged to remove the culture medium. Cells were resuspended in 4 ml of flow washing buffer (FWB) and centrifuged, and the supernatant was removed. Cells were counted, an aliquot of 1,000,000 cells was resuspended in 20 μl of FWB, and 5 μl of fluorescein labeled CD14 antibody was added to the cells for 20 min at 4°C. The cells were washed twice with 4 ml of FWB and then resuspended in 4 ml FWB. Cells were fixed with 200 μl of 1% paraformaldehyde for at least 30 min and then analyzed by flow cytometry (FACSARia; BD Biosciences).

Statistics. Statistical analyses were performed using GraphPad Prism (v. 5.03) and GraphPad InStat (v. 3.10). Tests for significance ($P$ values) were calculated using Student’s $t$ test for paired or unpaired data with or without Welch’s approximation to correct for unequal standard deviations when appropriate. Survival curves were analyzed using a contingency table.

RESULTS

Therapeutic proof of relevance. Nineteen cows were clinically diagnosed with mastitis (group B Streptococcus and Escherichia coli), and 9 were treated with pDAG (0.25 mg/kg of body weight, intramuscularly [i.m.], day 0 and day 3), and 10 received the standard of care (Polyflex brand of ampicillin; 5 mg/lb, i.m., for 3 days). The standard practice in the dairy industry is to measure the leukocyte count in the milk of cows as a diagnostic metric (normal milk contains $<500$ cells/μl). pDAG reduced the leukocyte count from 2,176 ± 347.5 to 510.6 ± 97.42 cells/μl (means ± standard deviations; $P = 0.0003$), whereas the antibiotic treatment group was unchanged ($P = 0.8$). These results are presented in Fig. 2A.

Salmonellosis is a significant diarrheal disease in humans. Whereas E. coli and group B streptococci (present in the bovine mastitis study) are extracellular pathogens, Salmonella enterica can infect macrophages during disease and are considered facultative intracellular pathogens. To determine if pDAG could modulate disease caused by an intracellular pathogen, we studied the effects of pDAG on S. enterica bacteremia. pDAG enhanced survival of mice in an experimentally induced S. enterica bacteremia model (Fig. 2B). Infected mice (5 × 10$^7$ CFU) either received a single dose of pDAG (0.25 mg/kg) or vehicle only (control) by subcutaneous injection at the time of infection. By day 10 all control mice died, whereas only 20% of pDAG-treated mice died ($P = 0.0001$).

acALY18 clears intracellular Chlamydia pneumoniae. Chlamydia pneumoniae causes an important community-acquired respiratory infection that persists in various human cells, including monocytes, where it is refractory to antibiotic treatment (17). The persistent form of C. pneumoniae has been implicated in the etiology of chronic diseases (19), and Appelt and colleagues recently showed that C. pneumoniae suppresses autophagy and subsequent

FIG 1 Chemical structure of pDAG and acALY18. The chemical structure of the 1-peptidyl-2-arachidonoyl-3-stearoyl-sn-glyceride natural product is shown. The 18-amino-acid peptide moiety is acALY18.
26 innate and adaptive immune genes were upregulated in infected monocytes versus infected/untreated cells. At 48 h postinfection, acALY18-treated (100 ng/ml) monocytes.

FIG 2. pDAG and acALY18 have broad-spectrum efficacies. (A) Comparison of pDAG treatment to the standard-of-care antibiotic treatment in 19 lactating dairy cows diagnosed with mastitis. The mean leukocyte counts in milk of the pretreatment groups were determined to not be statistically different ($P = 0.3965$), and neither were the standard deviations of the two groups ($F = 1.889; P = 0.3828$). The sampled data were derived from a population that followed a Gaussian distribution, tested by the Kolmogorov and Smirnov method and determined to have a normal distribution (KS, $-0.2; P > 0.01$). The mean pretreatment leukocyte count in the pDAG group ($n = 9$) was $2,176 \pm 347.5$ cells/ml, and 30 days posttreatment the count was $510.6 \pm 97.42$ cells/ml. **, $P = 0.0003$. The mean pretreatment leukocyte count in the antibiotic (Polypen brand of ampicillin) group ($n = 10$) was $2,681 \pm 453.2$ cells/ml, and 30 days posttreatment the count was $2,859 \pm 768.4$ cells/ml ($P = 0.8439$). (B) Treatment with pDAG enhanced survival of mice in an experimentally induced Salmonella enterica bacteremia. Swiss Webster mice ($n = 40$) were infected by intraperitoneal injection of $\sim 5 \times 10^8$ CFU of Salmonella enterica serovar Typhimurium. By the fourth day postinfection, mice in the group ($n = 20$) receiving a normal saline placebo treatment at the time of infection exhibited mortality. The rate of mortality rapidly increased over the next 6 days in this group and reached 100% by day 10 postinfection. By comparison, mice in the pDAG-treated group ($n = 20$) did not exhibit any mortality until day 8 postinfection (1 mouse), and by day 10 mortality was 20% (5 mice). The study was terminated on day 10. (C) THP-1 human monocytes ($1 \times 10^6$ cells) were infected at an MOI of 1 with C. pneumoniae ($1 \times 10^6$ IFU of strain AR-39) for 48 h. C. pneumoniae infected ~90% of the monocytes, as shown by staining with FITC-conjugated anti-Chlamyda antibody (Fitzgerald 61C75), counterstained with bis-Benzimide, and imaged at 40× magnification. (D) acALY18 (100 ng/ml without transfection reagent) was added to C. pneumoniae-infected THP-1 monocytes 24 h postinfection, and cells were incubated for an additional 24 h. acALY18-treated cells were imaged at 48 h postinfection for C. pneumoniae and showed ~12% of the cells were infected after acALY18 treatment.

lysosome-phagosome fusion to promote intracellular survival within the phagosome (3). We wanted to determine if acALY18 would override the escape mechanism of C. pneumoniae and induce clearance from infected monocytes.

Human THP-1 monocytes were infected (multiplicity of infection [MOI], 1) and incubated for 24 h, followed by addition of acALY18 (25, 50, and 100 ng/ml) and analysis 24 h posttreatment. THP-1 monocytes did not tolerate Lipofectamine well, necessitating a higher concentration of acALY18 in these experiments to ensure its intracellular delivery, as we have previously reported (34). A dose-response relationship was observed: ~90% of the untreated cells remained infected, and ~12% of the acALY18-treated cells remained infected at 100 ng/ml, ~35% at 50 ng/ml, and ~40% at 25 ng/ml. Figure 2C and D show the representative photomicrographs of infected, untreated monocytes and the infected, acALY18-treated (100 ng/ml) monocytes.

We examined gene expression from infected/acALY18-treated monocytes versus infected/untreated cells. At 48 h postinfection, 26 innate and adaptive immune genes were upregulated >4-fold in infected/treated cells, and 4 genes were downregulated at least 3-fold. Functional grouping of the increased gene expression included the following: (i) cytokines/chemokines, receptors, and signaling proteins (CCL2, INF1B1, INFGR1, IL-1β, IL1F5, IL1F7, IL1F8, IL1F9, IL1F10, IL1RN, IL-6, MYD88, TLR1, TLR10, TLR6, TLR8, and TNF); (ii) host defense (DEFB4, DMBT1, NCF4, NLRC4, and S100A12); (iii) antibacterial response (COLEC12, CYBB, and LY96); (iv) modulators of the tissue response to inflammation (ADORA2A, P2AFR, SERPINA1, SERPINE1, and TREM1). These results are presented in Table S1 of the supplemental material.

acALY18 activates the inflammasome. We postulated that acALY18 induces a signaling cascade that is at least in part mediated by the inflammasome (34). Inflammasome effector mechanisms have recently been reviewed (24), and it is clear that the inflammasome is a key regulator of the innate immune response to cellular injury and infection. The IL-1 family of cytokines are expressed as procytokines, and secretion is dependent upon activation of the cysteine protease caspase-1. IL-1β, IL-18, and IL-33, all IL-1 family cytokines, can act in an autocrine or paracrine manner, and their receptors are present in a variety of cell types, including fibroblasts and keratinocytes as well as tissue-resident immune cells (4).
acALY18-treated fibroblasts expressed 2.5-fold more active caspase-1 than untreated fibroblasts, and this activity was ablated by the caspase-1 inhibitor Z-YVAD(OMe)-FMK. acALY18 induced a 5-fold increase in secreted IL-1β, a 2.1-fold increase in secreted IL-18, and a 1.9-fold increase in secreted IL-33 relative to untreated fibroblasts (Fig. 3). The caspase-1 inhibitor significantly reduced acALY18-induced IL-1β and IL-18 secretion to concentrations less than that in untreated control cells and reduced IL-33 secretion to a concentration that was statistically equivalent to that secreted by untreated control cells. Data are presented as means of three separate experiments in duplicate ± the standard deviation.

acALY18 induces innate and adaptive immune gene transcription. We examined the specific inflammasome-related genes that were up- or downregulated more than 2-fold at 24, 48, and 72 h after treatment of primary human fibroblasts with acALY18 (3 ng/ml). At 24 h, 27 inflammasome-related genes were upregulated and 1 gene was downregulated, but none of these genes was found associated with an NLRP in many inflammasomes. NLRP3 is one of the most well-studied NLRPs and is generally believed to respond to damage-associated molecular patterns (DAMPs), like extracellular ATP, uric acid, or amyloid-β, as well as intracellular DAMPs, like reactive oxygen species (15, 32). Therefore, we examined the effects of acALY18, a presumptive DAMP, on fibroblasts from wild-type C57BL/6 mice and from ASC−/− and NLRP3−/− mice of the C57BL/6 background. Similar to the effects in human fibroblasts, acALY18 induced IL-1β, IL-18, and IL-33 secretion from C57BL/6 mouse fibroblasts and this was abrogated by Z-YVAD(OMe)-FMK. acALY18 did not induce significant secretion of IL-1β, IL-18, or IL-33 in the ASC−/− or NLRP3−/− fibroblasts, confirming that the effects of acALY18 are mediated, at least in part, by the NLRP3 inflammasome (Table 1).

FIG 3 acALY18 induces inflammasome-mediated cytokine secretion from primary human dermal fibroblasts. Normal primary human fibroblasts derived from a single patient were treated with Lipofectamine alone or with acALY18 (3 ng/ml)/Lipofectamine with or without 20 μM Z-YVAD(OMe)-FMK/Lipofectamine. Medium was collected after 24 h and assayed for secreted IL-1β, IL-18, and IL-33, and cells were recovered and assayed for caspase-1 activity. acALY18 induced a 2.5-fold increase in active caspase-1 relative to the untreated fibroblasts, and this was ablated in the presence of the caspase-1 inhibitor (top left). acALY18 induced a 5-fold increase in secreted IL-1β (top right), a 2.1-fold increase in secreted IL-18 (bottom left), and a 1.9-fold increase in secreted IL-33 (bottom right) relative to untreated fibroblasts. The caspase-1 inhibitor significantly reduced acALY18-induced IL-1β and IL-18 secretion when used alone. The caspase-1 inhibitor also reduced acALY18-induced IL-33 secretion to a concentration that that was statistically equivalent to that secreted by untreated control cells. Data are presented as means of three separate experiments in duplicate ± the standard deviation.
TABLE 1 acALY18 requires a functional inflammasome for induction of IL-1β, IL-18, and IL-33 secretion*

<table>
<thead>
<tr>
<th>Cytokine measured and mouse line</th>
<th>Cytokine secretion (pg/ml, ± SD)</th>
<th>P value (comparison)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-1β</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6</td>
<td>41.26 ± 3.04</td>
<td></td>
</tr>
<tr>
<td>C57BL/6 + acALY18</td>
<td>71.47 ± 0.23</td>
<td>0.0006 (vs C57BL/6)</td>
</tr>
<tr>
<td>C57BL/6 + YVAD</td>
<td>33.1 ± 3.17</td>
<td>0.14 (vs C57BL/6)</td>
</tr>
<tr>
<td>C57BL/6 + YVAD + acALY18</td>
<td>53.57 ± 1.22</td>
<td>0.02 (vs C57BL/6 + acALY18)</td>
</tr>
<tr>
<td>ASC−/−</td>
<td>22.17 ± 2.99</td>
<td></td>
</tr>
<tr>
<td>ASC−/− + acALY18</td>
<td>19.80 ± 2.65</td>
<td>0.59 (vs ASC−/−)</td>
</tr>
<tr>
<td>NLRP3−/− + acALY18</td>
<td>22.63 ± 1.45</td>
<td></td>
</tr>
<tr>
<td>NLRP3−/− + YVAD + acALY18</td>
<td>16.97 ± 2.20</td>
<td>0.09 (vs NLRP3−/−)</td>
</tr>
<tr>
<td><strong>IL-18</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6</td>
<td>51.63 ± 2.07</td>
<td></td>
</tr>
<tr>
<td>C57BL/6 + acALY18</td>
<td>63.23 ± 2.70</td>
<td>0.05 (vs C57BL/6)</td>
</tr>
<tr>
<td>C57BL/6 + YVAD</td>
<td>33.6 ± 1.27</td>
<td>0.004 (vs C57BL/6)</td>
</tr>
<tr>
<td>C57BL/6 + YVAD + acALY18</td>
<td>33.07 ± 0.47</td>
<td>0.002 (vs C57BL/6 + acALY18)</td>
</tr>
<tr>
<td>ASC−/−</td>
<td>33.53 ± 2.40</td>
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</tr>
<tr>
<td>ASC−/− + acALY18</td>
<td>35.76 ± 0.88</td>
<td>0.35 (vs ASC−/−)</td>
</tr>
<tr>
<td>NLRP3−/− + acALY18</td>
<td>32.77 ± 2.00</td>
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<tr>
<td>NLRP3−/− + YVAD + acALY18</td>
<td>31.13 ± 0.95</td>
<td>0.53 (vs NLRP3−/−)</td>
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<tr>
<td><strong>IL-33</strong></td>
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<tr>
<td>C57BL/6</td>
<td>143.97 ± 2.52</td>
<td></td>
</tr>
<tr>
<td>C57BL/6 + acALY18</td>
<td>159.10 ± 0.95</td>
<td>0.03 (vs C57BL/6)</td>
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<td>C57BL/6 + YVAD</td>
<td>118.13 ± 2.22</td>
<td>0.01 (vs C57BL/6)</td>
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<td>C57BL/6 + YVAD + acALY18</td>
<td>137.93 ± 1.76</td>
<td>0.02 (vs C57BL/6 + acALY18)</td>
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<tr>
<td>ASC−/−</td>
<td>79.20 ± 6.04</td>
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<tr>
<td>ASC−/− + acALY18</td>
<td>93.93 ± 2.51</td>
<td>0.13 (vs ASC−/−)</td>
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<tr>
<td>NLRP3−/− + acALY18</td>
<td>86.10 ± 5.15</td>
<td></td>
</tr>
<tr>
<td>NLRP3−/− + YVAD + acALY18</td>
<td>94.76 ± 3.49</td>
<td>0.22 (vs NLRP3−/−)</td>
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</table>

* Experiments were conducted in triplicate using mouse fibroblasts derived either from wild-type C57BL/6 control mice or the corresponding knockout, also on a C57BL/6 background. ASC, Apoptosis associated Speck-like protein containing a CARD; ASC−/−, the ASC knockout; NLRP3, the NOD-like receptor protein 3; NLRP3−/−, the NLRP3 knockout; YVAD, the caspase 1 inhibitor Z-YVAD(Ome)-FMK.

acALY18 induces antigen-specific IgM. A key feature of the innate immune response is production of opsonizing molecules, like complement. In primary human fibroblasts, acALY18 induced a 10-fold upregulation of C5 and C8 complement genes at 48 h (see Table S2 in the supplemental material). acALY18 promotes IL-33 secretion by fibroblasts, and IL-33 activates B1 cells in the mouse (1) to contribute to serum IgM levels (36). We examined IgM production in rabbits treated with either Freund’s complete adjuvant (FCA) as a positive control, Freund’s incomplete at levels significantly different from untreated cells. At 72 h, 39 genes were significantly upregulated, including six inflammasome platform genes (AIM2, NLRP3, NLRP4, NLRP5, NLRP9, and NOD2), and 36 genes were significantly downregulated, including apoptosis regulators (BCL2, BIRC2, and FADD). Other inflammasome-associated protein mRNAs were upregulated at 72 h, including the caspase recruitment domain family members CARD18 and CARD6 (214-fold and 1,600-fold, respectively) and Mediterranean fever pyrin (93-fold). Forty-eight genes associated with the innate and adaptive immunity were upregulated by 48 h, including caspase-1 and caspase-4 (9.9-fold and 5.8-fold, respectively), interleukin receptor-associated kinases 1 and 2 (26-fold, and 95-fold, respectively), MyD88 (5-fold), and NF-κB (27-fold). IL-1β mRNA expression was unchanged at 24 h but was upregulated nearly 50-fold at 48 h, and it declined to a 4-fold upregulation by 72 h. Several other cytokine genes were upregulated, including the type I interferons (IFN-α, 10.8-fold; IFN-β, 9.3-fold), IFN-γ (5.5-fold), IL-10 (17-fold), and IL-18 (2-fold), as were the genes for cellular defensive proteins, such as nitric oxide synthase (135-fold), C5 (10-fold) and C8 (10-fold), cathelicidin (10-fold), defensin-Bα (14-fold), and the lysosomal protease cathepsin B (34-fold). Clearly, the gene regulation that is induced by acALY18 enhances cellular defensive responses. It also appears that the inflammatory response to acALY18 reaches a maximum around 48 h and is downregulated by 72 h. The pharmacodynamic profiles for selected gene expression are depicted in Fig. 4, and summaries of the complete gene expression results are presented in Tables S2 and S3 of the supplemental material.

FIG 4 acALY18-induced gene expression reaches a maximum by 48 h. The expression levels of key inflammasome, innate, and adaptive immune genes in primary human fibroblasts were plotted at 24 h, 48 h, and 72 h after incubation with acALY18 (5 ng/ml [90% effective concentration]). Gene expression reached a maximum at 48 h and was significantly downregulated by 72 h. A complete tabulation of the gene expression levels for all genes measured is presented in Tables S2 and S3 of the supplemental material.
adjuvant (FIA) as a negative control, or acALY18 in FCA. FNN-21 is the universal tetanus toxin epitope, and it functions as an adjuvant; FNN-21 in FCA was also used as a mock control (37). The IgM titers in rabbits receiving acALY18 were increased by day 3, compared to rabbits receiving either FCA or FNN-21/FCA, and by day 5 this increase was 2-fold (P < 0.01). At days 10 to 14 acALY18 had increased the IgM titer by 3-fold (P < 0.001). Results are presented in Fig. 5.

Pharmacodynamics of acALY18. We evaluated the pharmacodynamics of modulating the host response to infection in a mouse model of a cutaneous MRSA infection. MRSA infection at 4.5 × 10⁷ CFU/mouse produced visible lesion formation in the skin within 72 h in 80% of the mice, and pathogen was detected in the circulation and spleens in 10% of the mice, suggesting that systemic MRSA infection had occurred. The mice that were treated with acALY18 at a presumptive minimum effective dosage (100 µg/kg; subcutaneous) had a >3-log reduction in pathogen load at the infection site 24 h postinfection (P < 0.05), and by day 10 the response was reduced >6 logs (P < 0.01) relative to the untreated mice (Fig. 6).

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In our study, we demonstrated increased myeloperoxidase at the infection site (Fig. 8), possibly a result of acALY18-induced secretion of IL-33 and activation of recruited neutrophils. IL-33 also enhances IgM production from tissue resident B1 cells (1, 36). Mice infected with MRSA and administered acALY18 either 24 h prior to infection or 24 h postinfection had enhanced IgM deposition at the infection site (Fig. 9). IL-33-driven IgM production and opsonization in addition to neutrophil activation may play a role in the enhanced clearance of MRSA in acALY18-treated mice.

In addition to increased IL-33 secretion, Thacker et al. demonstrated that human fibroblasts secrete IL-8 in response to acALY18 (34). IL-8 is a potent chemotactic chemokine in humans that is responsible for the recruitment of neutrophils. Mice do not produce IL-8, and the corresponding neutrophil-recruiting cytokine is KC/GRO/CXCL1. Miller and colleagues (26) established that IL-1R/MyD88-dependent KC/GRO/CXCL1 production in mouse skin cells promoted neutrophil and phagocytic pathogen clearance. We measured an increased and prolonged presence of KC/GRO/CXCL1 in the circulation of mice that were adminis-
tered acALY18 (Fig. 10A), and tissue histology showed enhanced myeloperoxidase activity (Fig. 8). *S. aureus* infections can activate the inflammasome (9, 27), mediating increased secretion of IL-1β/H9252 and IL-18, which is required for neutrophil recruitment (27). *S. aureus* also inhibits neutrophil function and can subvert the innate immune response (7, 38). The latter point is under investigation in our laboratories to determine if acALY18-induced inflammasome activation overrides *S. aureus* escape mechanisms in neutrophils.

Circulating cytokines and chemokines (monocyte chemoattractant protein 1 [MCP-1]/CCL2, IL-1β/H9252, IL-4, IL-6, KC/GRO/CXCL1, IL-10, IFN-γ/H9252, TNF-α/H9252, IL-12p40, and macrophage inflammatory protein 1α [MIP-1α]) were measured. IL-6, MCP-1/CCL2, KC/GRO/CXCL1, and IL-12p40 were the only cytokines/chemokines detected in the circulation of treated or untreated (control) mice over the 10-day study period (Fig. 10A to D). MCP-1/CCL2 was elevated in the serum of treated mice relative to control mice 1 day after infection, but this difference was not quite statistically significant (Fig. 10D). MCP-1/CCL2 remained detectable through day 10 postinfection in both groups.

Recruitment, activation, and differentiation of monocytes are key features of the innate immune response. Thacker et al. previously showed acALY18 induces the secretion of monocyte chemoattractant protein (CCL2) from human fibroblasts (34). The CCL2 gene was upregulated 18-fold at 48 h and significantly downregulated (204-fold) at 72 h (see Table S2 in the supplemental material). Mice treated with acALY18 24 h prior to infection had elevated levels of CCL2 in the circulation compared to control mice.
the treated mice. (Fig. 10D), thus suggesting enhanced monocyte recruitment in vehicle control group did not. All images were taken at 100 day 10 both of the acALY18-treated groups had IgM staining, whereas the not observed in mice treated 24 h prior to infection until day 5. However, at day 3 in mice treated 24 h postinfection and vehicle controls, whereas it was deposition of IgM was observed of MRSA skin lesions in mice treated 24 h prior to infection, 24 h postinfection, (Fig. 11).

acALY18 not only enhances recruitment of monocytes, but (Fig. 10D), thus suggesting enhanced monocyte recruitment in the treated mice.

In a separate in vitro experiment, we investigated activation of monocytes treated with conditioned medium from fibroblasts that were pretreated with acALY18, cocultured with acALY18-pretreated fibroblasts, or medium from untreated fibroblasts (control). THP-1 monocytes had a 2-fold increase in CD14 expression ($P = 0.0008$) when treated with the conditioned medium from fibroblasts previously treated with acALY18, compared to THP-1 monocytes treated with conditioned medium from the control fibroblasts. THP-1 monocytes that were cocultured with fibroblasts previously treated with acALY18 showed a 4-fold increase in CD14 expression ($P = 0.0002$), which was greater than levels observed in monocytes cultured in fibroblast-conditioned medium ($P = 0.008$). THP-1 monocytes also showed a 50% increase in the expression of CD69 when cocultured with the acALY18-pretreated fibroblasts ($P < 0.05$). These results suggest that acALY18 not only enhances recruitment of monocytes, but also that recruited monocytes differentiate into macrophages (Fig. 11).

**FIG 9** IgM (green) staining of MRSA skin lesions. Representative IgM stains of MRSA skin lesions in mice treated 24 h prior to infection, 24 h postinfection, and vehicle controls during the 10-day study. Deposition of IgM was observed at day 3 in mice treated 24 h postinfection and vehicle controls, whereas it was not observed in mice treated 24 h prior to infection until day 5. However, at day 10 both of the acALY18-treated groups had IgM staining, whereas the vehicle control group did not. All images were taken at 100× magnification.

**DISCUSSION**

For more than 75 years the therapeutic paradigm for infectious diseases has been to administer a toxin that is more toxic to the pathogen than it is to the patient. Antibiotics have been of unquestionable benefit in terms of lives saved and the alleviation of suffering from infectious diseases, but this benefit has not been without unanticipated consequences. Present efforts toward new antibiotic drug development have not yielded NMEs to adequately address the current need and have arguably contributed to the current crisis of emergent antibiotic resistance.

The development of new anti-infective agents to address the epidemic of emerging antibiotic-resistant infections is a pressing unmet medical need. The strategy of modulating the host response to infection holds promise for development of new anti-infective drugs that are safe, effective, and less likely to promote resistant strains of pathogens (21). However, if we are to embark on this path toward the development of a new therapeutic strategy, that effort should be predicated upon a deeper understanding of mammalian resistance and tolerance mechanisms.

Mammals and their microbiota have symbiotically coevolved over millions of years. Potential pathogens, such as *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Neisseria meningitidis*, colonize specific anatomical compartments and are held in check by tolerance mechanisms. Further, at any given moment we are challenged by infections from any number or type of environmental pathogens, but the vast majority of these infections do not progress to disease, due to the effectiveness of our highly evolved resistance mechanisms.

Pathogens have also evolved a variety of defensive mechanisms to escape detection and eradication, and it is becoming increasingly evident that many of the chronic idiopathic diseases are clinical manifestations of an inflammatory state maintained by the cellular response to the presence of a persistent infection. It would seem prudent, therefore, to understand the host-microbe defense and escape mechanisms as a means to develop pharmaceutical strategies for drug intervention in infectious diseases and possibly many of the chronic diseases as well.

Our laboratories have been studying the host response to pathogen infection in order to develop a pharmaceutical intervention that has a broad spectrum in its effect and that can abrogate an infection from progressing to disease. Herein we have described an NME (acALY18) that can be administered to augment a host defense mechanism via the NLRP3 inflammasome and induce clearance of either a Gram-positive or Gram-negative bacterium or override the escape mechanism of an intracellular bacterial pathogen.

In an experimentally induced MRSA skin infection, the downstream effect of acALY18 modulation of inflammasome signaling is an enhanced innate immune response. These effects include cytokine- and chemokine-mediated activation of tissue resident immune cell phenotypes, recruitment and activation of professional phagocytic cells, and production of IgM to opsonize the invading pathogen, culminating in enhanced pathogen clearance.

acALY18-induced immune gene expression reaches a maximum by 48 h, and by 72 h it is significantly downregulated. The cytokine and chemokine expression profiles in the sera of MRSA-infected mice treated with acALY18 were consistent with the gene expression profile. This observation suggests that the cellular response to acALY18 administration is short-lived and is subject to...
inherent negative feedback control mechanisms and/or a short in vivo half-life for acALY18. These results also suggest that administration of a second dose of acALY18 at 48 to 72 h hours after the initial dose might prolong the innate immune response and enhance the efficacy for pathogen clearance. More detailed pharmacokinetic and pharmacodynamic studies will be required to answer these important questions.

Nevertheless, in light of the broad-spectrum effect, the pharmacodynamic properties of acALY18-mediated inflammasome modulation seem to be well-suited for adjunctive treatment or prophylactic treatment for antibiotic-resistant infections. Ongoing pharmacokinetic, toxicity, and pharmacological safety studies will establish the viability of this strategy and pave the way for first-in-humans clinical evaluations.

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FIG 10 acALY18 promotes immune cell recruitment and activating cytokines and chemokines. Serum cytokines/chemokines elevated over the 10-day study for mice that were treated 24 h postinfection compared to vehicle-treated mice. Data are presented as means \( \pm\) SEM of \( n = 3 \) for each sampling interval on study days 1, 3, 5, and 10. (A) Serum IL-12p40 levels were not significantly elevated in the serum of treatment (red *) or control (blue *) mice until day 3, and levels were comparable thereafter (\( P > 0.05 \)). (B) Serum IL-6 levels in mice receiving acALY18 (red *) or the vehicle control (blue *). By day 3 serum levels of IL-6 in the treated mice were significantly elevated relative to untreated control mice (*, \( P < 0.01 \)) and remained statistically elevated through day 5 (**, \( P < 0.05 \)). By day 10 postinfection IL-6 in all mice had returned to baseline levels. (C) Serum CXCL1 levels in mice receiving acALY18 (red *) or the vehicle control (blue *). By day 3 serum levels of CXCL1 in treated mice were significantly elevated relative to untreated control mice (*, \( P = 0.0002 \)) and remained significantly elevated through day 5. CXCL1 remained elevated in the serum of treated mice through day 10 but had returned to baseline levels in the untreated mice (**, \( P < 0.05 \)). (D) MCP-1/CCL2 levels were elevated in the serum of treated mice (red *) relative to the untreated control mice (blue *) 1 day after infection, but this difference was not quite statistically significant (\( P = 0.06 \)) and remained detectable through day 10 in both groups (\( P > 0.05 \)).

FIG 11 acALY18-treated fibroblasts induce differentiation of recruited monocytes. Primary human fibroblasts were cultured in the presence of acALY18 (3 ng/ml) or the vehicle alone (control) for 48 h. In one experiment, the conditioned medium from the fibroblast culture was collected and added to cultured THP-1 monocytes for 72 h. In a separate experiment, THP-1 monocytes were added to the fibroblast culture pretreated with acALY18 (cocultured) along with fresh medium and incubated for an additional 72 h. Monocytes from both experiments were collected and analyzed for CD14 (A) and CD69 (B) expression by flow cytometry. *, \( P = 0.0002 \); **, \( P = 0.0008 \); ***, \( P < 0.05 \).
conducted the bacteremia study of mice, and Lampire Biologicals prepared the rabbit polyclonal anti-acALY18 sera.

J.D.T. is the principal stockholder and President/Chief Science Officer for TherimuneX Pharmaceuticals, Inc. S. Sassi-Gaha, M. Purohit, C. M. Artlett, and R. F. Rest are employees of Drexel University College of Medicine, which has a financial interest in TherimuneX Pharmaceuticals, Inc.

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